·Basic Research·

Effects of dexamethasone and HA1077 on actin cytoskeleton and β-catenin in cultured human trabecular meshwork cells

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Abstract

• **AIM: To investigate the effects of dexamethasone (DEX) and 1-(5-isoquinolinesulfonyl)-homopiperazine (HA1077) on actin cytoskeleton and β-catenin in cultured human trabecular meshwork (HTM) cells.**

• **METHODS: The HTM cells were separated from human eyeball and cultured *in vitro*. They were divided into control group, DEX (1×10^{-6} mol/L) group, HA1077 (3×10^{-5} mol/L) group, and DEX (1×10^{-6} mol/L) and HA1077 (3×10^{-5} mol/L) group. Actin cytoskeleton and β-catenin in HTM cells of the four groups were examined by immunofluorescence and Western blot analyses.**

• **RESULTS: In DEX group, there were reorganization of actin cytoskeleton and formation of cross linked actin networks (CLANs), which were partially reversed in DEX and HA1077 group. DEX treatment also induced an increased expression of β-catenin, which was obviously reduced in DEX and HA1077 group. Meanwhile, the cultured HTM cells in HA1077 group had lower expression of β-catenin than that in the control group.**

• **CONCLUSION: Our results show that HA1077 can reverse the changes of actin organization and expression of β-catenin induced by DEX in cultured HTM cells, suggesting that HA1077 may play an important role in increasing outflow and reducing intraocular pressure.**

• **KEYWORDS:** HA1077; trabecular meshwork cell; dexamethasone; actin cytoskeleton; β-catenin
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INTRODUCTION

Elevated intraocular pressure (IOP) is one of risk factors for primary open angle glaucoma (POAG). For most of POAG patients, an abnormally high aqueous humor outflow resistance in the juxtacanalicular (JXT) region of the trabecular meshwork (TM) could lead to increase of IOP and in fibrillar extracellular matrix (ECM)[1].

This ocular hypertension is due to increased hydrodynamic resistance to the drainage of aqueous humor through specialized outflow tissues could result in ocular hypertension. It is generally accepted that the structure changes of actin cytoskeleton, associated cell-cell junctions and cell-ECM interactions would alter outflow facility [2]. Actin cytoskeleton is involved in maintaining cell shape, cell-cell and cell-ECM interactions. Aqueous humor drainage could be influenced by intercellular adherens junctions and cell-ECM [3]. A variety of evidences have been shown that cytoskeleton plays an important role in determining aqueous outflow resistance [2,4]. A number of studies have documented certain agents designed to lower IOP for glaucoma treatment[2,4], some of which could be capable of increasing aqueous outflow by either altering the actin cytoskeleton or targeting ECM [3,5-9]. To date, the actin cytoskeleton and associated cellular-adhesion proteins have presented attractive research directions for novel therapeutic methods for glaucoma[3,8-9].

It is reported that protein kinase inhibitor, 1-(5-isoquinolinesulfonyl)-homopiperazine (HA1077), can

increase outflow facility or decrease IOP through interfering with the actin cytoskeleton, associated cellular-adhesion and ECM [7]. However, the mechanisms for decreasing IOP and increased outflow have not been fully studied and may be involved in multiple signaling pathways. Dexamethasone (DEX) has been reported to be used to induce high IOP with increased aqueous humor outflow resistance associated with biochemical changes in the TM through noncanonical Wnt signaling [10-11]. β-catenin have been shown to play a key role in Wnt signaling pathway, therefore, we aimed to evaluate the effects of HA1077 on the expression level of actin cytoskeleton and β-catenin in cultured human TM cells treated with DEX, and to explore the mechanism of β-catenin participating in the pathogenesis of POAG.

SUBJECTS AND METHODS

Cell Culture The human eyeballs, used to separate human trabecular meshwork (HTM) cells, were graciously donated by a deceased 29-year-old Han Chinese male from Hospital of University of Electronic Science and Technology of China and Sichuan Provincial People's Hospital. All procedures in this study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Boards of the Hospital of University of Electronic Science and Technology of China & Sichuan Provincial People's Hospital. Written informed consents were obtained from his relative and he was excluded to suffer from glaucoma and have a family history of glaucoma and other eye diseases.

The eyeballs obtained within 24h after death were placed in sterile dish and transected 5 mm posterior to the limbus. Under an operating microscope, the vitreous, the lens, the uvea and the pectinate ligaments were carefully removed away. The TM were gently separated with jeweler's forceps and cut into pieces, and then placed in culture bottle or 24-well culture plates, incubated at 37℃ in DMEM/F12 (Hyclone) containing 20% fetal bovine serum (Invitrogen), and antibiotics (100 units/mL penicillin G and 100 μg/mL streptomycin sulfate, Invitrogen). The growth medium was changed twice a week and the cells were treated with 0.25% trypsin (Hyclone) and passaged after confluence. All the experiments used third-passaged. The cells were identified by using morphology and immunohistochemistry. ECM staining including neuron-specific enolase (NSE), fibronectin (FN), laminin (LN), collagen I (CAI), collagen IV (CAIV) were used to evaluate cultured cells.

Treatment by Dexamethasone and 1 -(5 -isoquinolinesulfonyl) -homopiperazine The third passage HTM cells were plated in culture flask and on glass cover slips which were pre-coated with poly-l-lysine (Invitrogen. DEX (Sigma) was dissolved with 100% ethanol (Hyclone). HA1077 (Sigma) was dissolved with DMSO (Invitrogen). According to previous study [7,12], the duration and dose of treatment with DEX and HA1077 were optimized in this study. The HTM cells were divided into four groups: control group; DEX group, 1×10^{-6} mol/L DEX was added to the culture media for 12d; DEX and HA1077 group, after 7d treatment with 1×10^{-6} mol/L DEX, 3×10^{-5} mol/L HA1077 was added to the culture media for 5d co-treatment; HA1077 group, after cells were cultured for 7d, 3×10^{-5} mol/L HA1077 was added to the culture media for 5d. Each group had six replicas. Phase-contrast microscopy (Leica) was used for observing cell morphology changes.

Immunofluorescence After fixing with 2% neutral buffered paraformaldehyde (Sigma) in 0.1 mol/L PBS, the cultured cells were permeabilized. Then, the cells were incubated with primary antibody, appropriate fluorescence conjugated IgG secondary antibody and DAPI (Sigma) staining. Mouse anti-human β-catenin monoclonal antibody, Goat anti-mouse immunoglobulin G Rhodamine (TRITC) conjugate and Goat anti-Rabbit Alexa Fluor ® 488 were purchased from Santa-Cruz. FITC-phalloidin used for the fluorescent labeling of actin cytoskeleton was purchased from Sigma. Rabbit anti-human NSE polyclonal antibody, rabbit anti-human FN polyclonal antibody, mouse anti-human LN monoclonal antibody, mouse anti-human CAI monoclonal antibody, mouse anti-human CAIV monoclonal antibody were purchased from Shanghai Shenggong biotechnology Co., LTD. Cell images were captured with a fluorescence microscope (Leica).

Western Blot Analysis Cells were homogenized with radio immunoprecipitation (RIPA) buffer and extracted proteins were transferred to polyvinyl difluoride (PVDF) membrane (Bio-Rad), which were incubated with primary antibody mouse anti-human β-catenin monoclonal antibody (Sigma, 1:1000) and monoclonal Anti-GAPDH-peroxidase antibody produced in mouse (Sigma, 1:15000) overnight at 4℃. Then, the membrane was followed to incubate with a secondary antibody HRP-labeled goat anti-mouse IgG (Invitrogen, 1:10000) for β-catenin at room temperature for one hour. Enhance chemiluminescence (Amersham, Bucks, UK) was used to detected the chemical signals.

RESULTS

Culture and Feature of Human Trabecular Meshwork Cells Ten to thirteen days after migration from the explants, the primary cultured HTM cells appeared diverse irregular morphology, such as spiky and filopodial projections (Figure 1A, 1B). Primary cultured HTM cells formed a confluent and flat monolayer with extensive intercellular contacts 3-4wk later separated from TM (Figure 1C). All the cells were positive with FN, LN, NSE, CAI and CAIV by immunostaining, suggesting that the primary cultured cells have the features of HTM cells reported previously[12].

Effects of Dexamethasone and 1-(5-isoquinolinesulfonyl)-homopiperazine on the Morphology of Human Trabecular Meshwork Cells The cultured HTM cell in

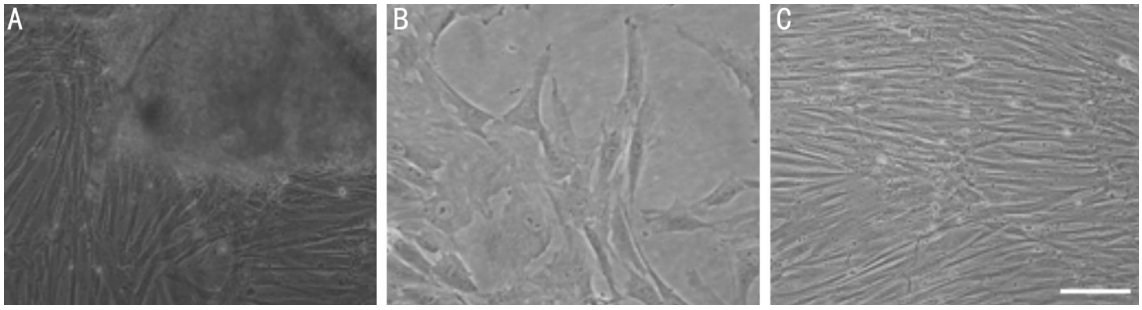

Figure 1 Primary cultured HTM cells A: Primary cultured HTM cells migrated from tissue; B: Primary cultured HTM cells appeared diverse irregular morphology, such as spiky and filopodial projections; C: Primary cultured HTM cells formed a confluent and flat monolayer with extensive intercellular contacts. Scale bars: 50 μm.

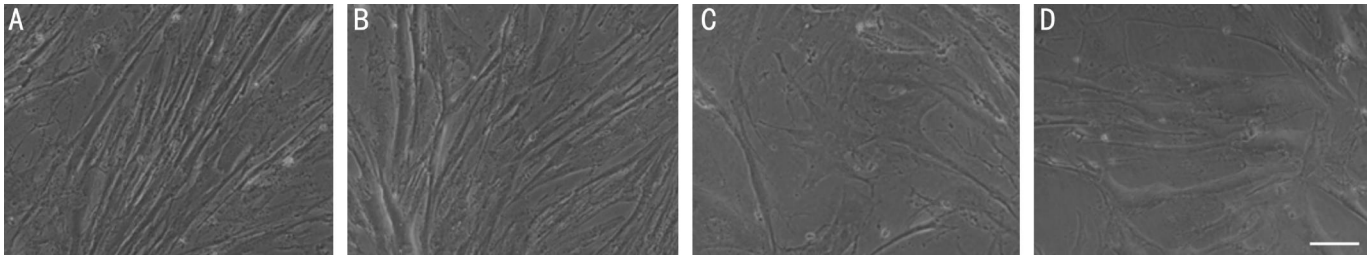

Figure 2 Morphology of cultured HTM cells treated with DEX and HA1077 A: Control group; B: DEX group; C: DEX+HA1077 group; D: HA1077 group. Scale bars: 50 μm.

DEX group appeared similar morphology in size, orientation and distribution with the cells in control group (Figure 2A, 2B). The cells in DEX and HA1077 group became loose, shrinkage and round, and markedly separated from each other (Figure 2C). The cells in HA1077 group showed the same morphology as the cells in DEX and HA1077 group (Figure 2D).

Effects of Dexamethasone and 1–(5–isoquinolinesulfonyl)–homopiperazine on Actin Cytoskeleton and β–catenin in Human Trabecular Meshwork Cells The cells in control group contained stress fibers, peripheral cortical actin, and minor actin tangles. Fluorescent staining of actin cytoskeleton showed there was numerous thick stress fibers mainly aligned along the longitudinal axis of the cells (Figure 3A). In DEX group, The microfilaments were reorganized in the cells and many of these cells developed crosslinked actin networks (CLANs). They surrounded the nucleus and eventually involved in the entire actin cytoskeleton (Figure 3B). Most of these cells had a perinuclear, geodesic dome-like pattern of CLANs except some cells with stress fibers. About half of these cells had CLANs from a small perinuclear pattern to a network encompassing the entire cell. The latter microfilament network "pushed" the actin filaments around the cell, with few microfilaments in the center. More DEX-treated cells had CLANs with cross-linked networks, compared to untreated TM. In DEX and HA1077 group，a reorganization of actin cytoskeletal and a formation of CLANs in TM cells treated by DEX were partially reversed. CLANs was reduced and part of them was degraded (Figure 3C). In HA1077 group, the cells were similar in actin cytoskeleton and actin microfilament patterns with untreated cells (Figure 3D).

β-catenin located around the periphery of normal and untreated TM cells, and distributed in cell membrane of cells linked together, just like the lace in the place of intensive cell growth (Figure 4A). After treatment with DEX for 12d, the expression of β-catenin was remarkably increased, compared to the cells in control group (Figure 4B). In DEX and HA1077 group, the expression of β-catenin in treated cells was reversed, compared to that in DEX and HA1077 group (Figure 4C). In HA1077 group, the expression of β-catenin was similar with that in control group (Figure 4D).

Western blot analyses showed that HA1077 obviously reversed the expression of β-catenin protein, which was remarkably induced in DEX group. The expression of β-catenin in DEX and HA1077 groups was decreased, compared to that in DEX group and control group (P <0.01), but the expression of β-catenin was higher than that in HA1077 group (P <0.01). Finally, the expression of β-catenin in HA1077 group was also reduced remarkably, compared to that in untreated cells (P <0.01, Figure 5).

DISCUSSION

TM, is the major site for regulating aqueous humor outflow at the chamber angle of the eye. Actin microfilaments play a key role in controlling cell functions, such as cell shape, proliferation, mobility/movement, cell-cell and cell-matrix junctions, polarity, and intracellular organization[3].

Consistent with the previous studies[10-11,13-15], we observed that DEX could alter the actin cytoskeleton and reorganize TM cytoskeleton to form CLANs in cultured HTM cells in this study. Clark *et al*'s study [16] showed that DEX treatment not only altered the actin cytoskeleton in cultured TM cells, but also lead to an elevated IOP in several eyes with more actin

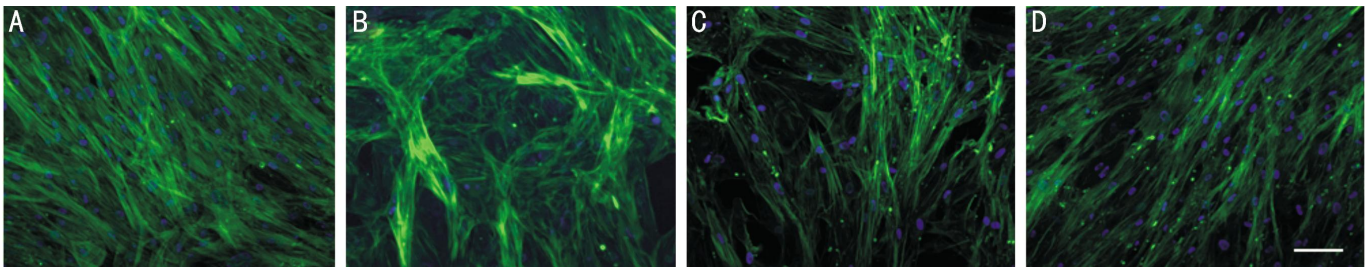

Figure 3 Fluorescent images of the effects of HA1077 and DEX on actin cytoskeleton in HTM cells A: Control group; B: DEX group; C: DEX+HA1077 group; D: HA1077group. Actin cytoskeleton was stained with green. Scale bars: 50 μm.

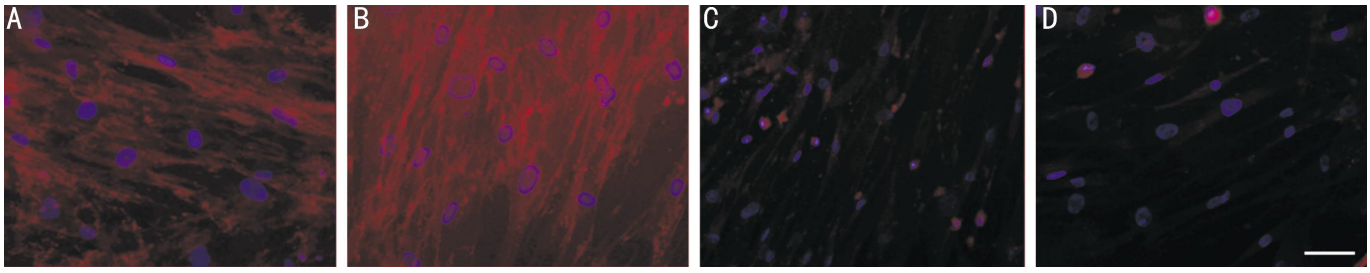

Figure 4 Fluorescent images of the effects of HA1077 and DEX on β-catenin in HTM cells A: Control group; B: DEX group; C: DEX+HA1077 group; D: HA1077 group. F-actin was stained with green. Scale bars: 50 μm.

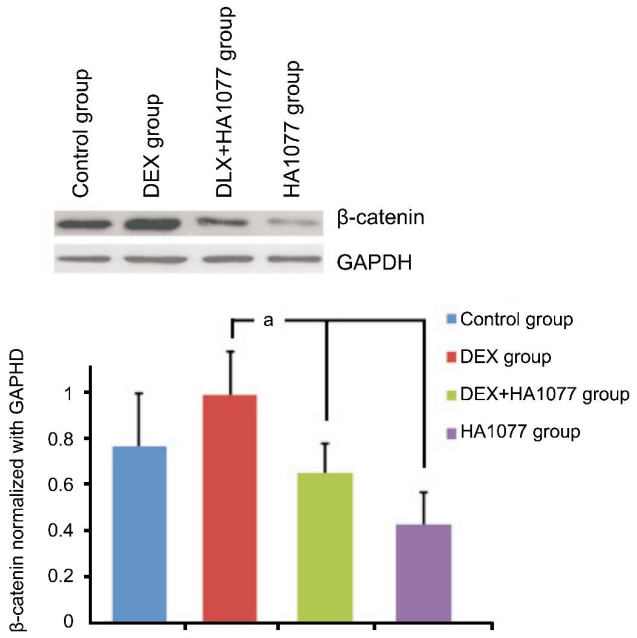

Figure 5 Western blot analysis of β-catenin in HTM cells [a] $P<0.05$.

tangles and the formation of CLANs in tissue may, therefore, interfere with the ability of TM cells for responding to stimuli such as stretch could be interfered by CLAN and F-actin tangle formation in the glaucomatous TM and glucocorticoid-treated TM [15-18]. HA1077, an anti-vasospastic compound, has been previously reported to act as a vasodilator *in vivo* to inhibit smooth muscle contraction and to change various cellular behaviors [19]. This compound has also been shown to increase outflow facility or decrease IOP through interfering with the actin cytoskeleton, associated cellular-adhesion and ECM [7]. Our data indicated that HA1077 greatly reversed microfilaments reorganization induced by DEX, reduced the CLAN structure formation and degraded part of the CLAN structure. Therefore, HA1077 could be used to alter TM cell actin microfilaments, disrupt actin and associated cellular adhesions, and thus increase outflow facility and lower IOP.

In this study, the cultured HTM cells became loose and intercellular gap widened obviously after HA1077 treatment. The broadening of cell-cell space is possible to make the outflow less obstructed [20]. The β-catenin plays an important role in cadherin-mediated cell adhesions and regulating nuclear transcriptional activity in the Wnt signaling pathway[21]. Wnt signaling have been reported to play a key role in different biologic processes, including cell differentiation, development, regulation, and apoptosis. More and more evidence showed that IOP could be regulated in Wnt signaling [22-23]. Not only does β-catenin function as an adherens junction adhesion protein, but also it is an essential mediator of the canonical Wnt signaling pathway. In this study, DEX group increased β-catenin expression, but HA1077 obviously inhibited β-catenin expression in cultured HTM cells. The ability of HA1077 reversed and decreased β-catenin expression increased by DEX treatment, suggesting that HA1077 could reduce the connection between cells, the adhesion between cells and ECM, leading to lower aqueous outflow resistance, thus lowering IOP.

This study used primary cultured HTM cells and observed that HA1077 could reverse the changes of actin organization and expression of β-catenin in the HTM cells induced by DEX. This result may help to elucidate the mechanism of action of HA1077 in increasing outflow and reducing IOP, and provide a novel therapeutic method for high tension glaucoma. Further work for the mechanism is needed before this approach can be used for the clinical treatment.

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